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A Specific Multi-Nutrient Diet Reduces Alzheimer-Like Pathology in Young Adult $A\beta PP_{swe}/PS1_{dE9}$ Mice

Laus M. Broersen[a,*], Almar A.M. Kuipers[a], Martin Balvers[a], Nick van Wijk[a], Paul J.M. Savelkoul[a], Martijn C. de Wilde[a], Eline M. van der Beek[a], John W.C. Sijben[a], Robert J.J. Hageman[a], Patrick J.G.H. Kamphuis[a,b] and Amanda J. Kiliaan[c]
[a]*Nutricia Advanced Medical Nutrition, Danone Research, Centre for Specialised Nutrition, Wageningen, The Netherlands*
[b]*Utrecht Institute for Pharmaceutical Sciences (UIPS), University Utrecht, Utrecht, The Netherlands*
[c]*Department of Anatomy, Radboud University Nijmegen Medical Centre, Donders Institute for Neuroscience, Nijmegen, The Netherlands*

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Abstract. Diet is an important lifestyle factor implicated in the etiology of Alzheimer's disease (AD), but so far it is not fully elucidated to which nutrients the suggested protective effect of diet can be attributed. Recent evidence obtained in the amyloid-β 1-42 ($A\beta_{42}$) infusion model in rats has shown that a multi-nutrient intervention known as Fortasyn™ Connect (FC) may protect the central cholinergic system against $A\beta_{42}$-induced toxicity. FC comprises the nutritional precursors and cofactors for membrane synthesis, viz. docosahexaenoic acid (DHA), eicosapentaenoic acid, uridine-mono-phosphate (UMP), choline, phospholipids, folic acid, vitamins B6, B12, C, E, and selenium. In order to investigate whether the combined administration of these nutrients may also affect AD-like pathology, we now evaluated the effects of the FC diet intervention in the transgenic $A\beta PP_{swe}/PS1_{dE9}$ mouse model with endogenous Aβ production. In addition we evaluated the effects of diets containing the individual nutrients DHA and UMP and their combination in this model. Between the age of 3 and 6 months, FC diet decreased brain Aβ levels and amyloid plaque burden in the hippocampus of AβPP/PS1 mice. The FC diet also reduced ongoing disintegrative degeneration in the neocortex, as indicated by Amino Cupric Silver staining. Although all three DHA-containing diets were equally effective in changing brain fatty acid profiles, diets differentially affected amyloid-related measures, indicating that effects of DHA may depend on its dietary context. The current data, showing that dietary enrichment with FC reduces AD-like pathology in AβPP/PS1 mice, confirm and extend our previous findings in the $A\beta_{42}$ infusion model and favor the combined administration of relevant nutrients.

Keywords: AβPP/PS1 transgenic mice, Alzheimer's disease, amyloid-β, degenerative staining, DHA, Fortasyn Connect, nutrition, plaque burden, souvenaid, UMP

INTRODUCTION

Diet is an important lifestyle factor implicated in the etiology of dementias including Alzheimer's disease (AD). Specific dietary patterns are associated with lower risks of developing dementias [1–5], and

*Correspondence to: Laus M. Broersen, Nutricia Advanced Medical Nutrition, Danone Research, Centre for Specialised Nutrition, PO Box 7005, 6700 CA Wageningen, The Netherlands. Tel.: +31 317 467 854; Fax: +31 317 466 500; E-mail: laus.broersen@nutricia.com.

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adherence to complex dietary compositions has been associated with better mental and physical health [6] and reduced risk of developing AD [7, 8]. The potentially protective effects of diet, however, cannot easily be attributed to the actions of individual nutrients, as a variety of systematic reviews conclude that single-nutrient interventions are predominantly non-successful [9–12]. Presumably, the combined intake of such nutritional components is required in order to reach efficacy.

A striking example of the added value that the simultaneous enrichment of nutrients can have comes from a series of experiments showing that the combination of the phospholipid precursors docosahexaenoic acid (DHA) and uridine-mono-phosphate (UMP) can act synergistically in stimulating membrane phospholipid synthesis, increasing dendritic spine density, and improving learning and memory [13–15]. The nutrients work by increasing the substrate-saturation of low-affinity enzymes that synthesize the phospholipids [16]. All these observations were made in healthy rodents, but the effects reported are considered to be very relevant for AD, in which brain phospholipids are reduced [17, 18] and which is characterized by a loss of synaptic connections [19, 20] thought to underlie the loss of cognitive functioning. In line with this suggestion, we recently reported that a multi-nutrient composition called Fortasyn™ Connect (FC) protected the central cholinergic system against amyloid-β 1-42 ($A\beta_{42}$)-induced toxicity in a membrane toxicity model of AD, the $A\beta_{42}$ infused rat [21]. Based on this, we were interested to see whether combined administration of nutrients that affect membrane synthesis and composition may also affect endogenous membrane-bound processes relevant to AD, such as Aβ production and amyloid plaque formation.

The aim of the present study was to investigate the effects of nutritional interventions on the development of AD-like pathological changes in female transgenic AβPP/PS1 mice. These female mice show Aβ plaque formation as early as 4–5 months of age [22, 23], which is sensitive to dietary manipulations [24]. We carried out two experiments in which dietary interventions were started at the age of 3 months and continued for another 3 months. In both experiments, effects of diets were assessed on brain composition and pathology, using Aβ ELISA, fatty acid analysis, as well as immunohistochemical techniques to assess amyloid plaque burden ($A\beta_{42}$ stain) and ongoing degenerative burden (deOlmos Amino Cupric Silver stain [25, 26]).

In a first experiment we evaluated the effects of the multi-nutrient FC composition that was designed to stimulate synaptic membrane formation, comprising a full set of precursors and cofactors for membrane synthesis, including UMP, DHA, eicosapentaenoic acid (EPA), choline, vitamin B6, vitamin B12, folic acid, phospholipids, vitamin C, vitamin E, and selenium. From these nutrients, UMP, DHA, and choline are all precursors for the Kennedy cycle for phospholipid synthesis [16, 27]. Dietary EPA contributes as a precursor to raise brain levels of DHA [28, 29]. The phospholipids act both as precursors and cofactors, by providing choline and diacylglycerol (DAG) for the Kennedy cycle and by enhancing the availability of DHA, respectively. The remaining cofactors, i.e., the B-vitamins and the antioxidants, are necessary to enable and support the biochemical processes involved in phospholipid synthesis and/or to increase the availability of the membrane precursors, either by stimulating the endogenous synthesis or by reducing their degradation (e.g., [30, 31]). In addition, selenium may enhance PC synthesis by increasing the activity of a key enzyme in the Kennedy cycle, CDP-choline:DAG cholinephosphotransferase [32]. Levels of choline, phospholipids, and polyunsaturated fatty acids like DHA are known to be decreased in the brains of AD patients [17, 18, 33], and epidemiological studies have reported that AD patients also have lower plasma levels of DHA, folic acid, vitamin B12, vitamin E, and vitamin C as compared to age-matched controls [34–36]. The effects of the FC diet intervention were studied both in AβPP/PS1 mice and their wild type littermates.

In a second experiment we evaluated the effects of diets that were supplemented with either DHA or UMP or the combination of these two nutrients in transgenic mice. DHA may reduce Aβ release by directing amyloidogenic processing of Aβ protein precursor (AβPP) toward non-amyloidogenic processing [37]. DHA has been evaluated before in various transgenic mouse models of AD pathology [24, 38, 39], but has not always been found to affect amyloid production or plaques [40, 41]. It has been suggested that the efficacy of DHA in reducing amyloid plaque formation may depend on its dietary context [42]. UMP has not been evaluated before in transgenic mouse models of AD, but has repeatedly been shown to act synergistically in combination with DHA on structural brain changes in healthy rodents [15, 16]. We now tested the combination of DHA and UMP to see whether these nutrients would also show synergistic effects on brain parameters in AβPP/PS1 mice.

MATERIALS AND METHODS

Animals and dietary interventions

The $A\beta PP_{swe}/PS1_{dE9}$ founders were obtained from Johns Hopkins University, Baltimore, MD, USA (Borchelt and Jankowsky, Department of Pathology) and a colony was established at the Radboud University Nijmegen Medical Centre, The Netherlands. The mice had been created by co-injection of chimeric mouse/human AβPPswe (mouse AβPP695 harboring a human Aβ domain and mutations K595N and M596L linked to Swedish familial AD pedigrees) and human PS1-dE9 (deletion of exon 9) vectors controlled by independent mouse prion protein promoter elements. This line (line 85 [22]) was originally maintained in a hybrid background by backcrossing to C3HeJ × C57Bl6/J F1 mice. For the present work, the breeder mice were backcrossed to C57Bl6/J for 9 generations.

Female AβPP/PS1 transgenic mice and wild type littermate controls were housed in groups of 4–6 animals per cage. The animals were kept in a controlled environment, with temperature at 20–22°C, humidity at 50–60%, and lights on between 07:00 and 19:00 h. Food and water were freely available throughout the study. Some animals died for unknown reasons during the study, and were discarded from the experiment.

At the age of 3 months, the mice were subjected to the experimental diets for a period of 3 months, as indicated in Table 1. In experiment A, both wild type and transgenic mice were fed with either a Control diet or a FC diet. In experiment B, groups of transgenic mice were fed DHA, UMP, or DHA + UMP diets. Experiment B was conducted to test the individual and combined effects of DHA and UMP supplementation. The diets differed in composition with regard to the fat blends used, as well as a number of supplemented nutrients as indicated in Tables 2 and 3. The source of DHA was fish oil (tuna, Numega, and EPA-28SP; Lithos Foods). All diets were isoenergetic, were based on the Control diet, fulfilled all dietary requirements, and were manufactured and pelleted by Research Diet Services (Wijk bij Duurstede, The Netherlands). All diets were stored at −20°C until use, in order to prevent oxidation of lipids. Reanalysis of the diets at the end of the study confirmed that all fatty acids were still present in the original amounts.

All animal experimental protocols were conducted in accordance with European and Dutch laws and institutional guidelines and approved by the local ethics committee (DEC Consult, Bilthoven, The Netherlands).

Table 1
Overview of groups of mice and the experimental diets for experiments A and B

Experiment	Genotype	Diet	# mice
A	Wild type	Control	11
		FC	10
	Transgenic	Control	7
		FC	7
B	Transgenic	Control	7
		DHA	5
		UMP	8
		DHA + UMP	7

Tissue preparation

At the age of 6 months, mice were perfused transcardially with ice-cold saline containing 5.8 mM EDTA under deep sodium pentobarbital anesthesia. Brains were rapidly removed and divided at the midline. The left hemisphere was snap frozen in liquid nitrogen and stored at −80°C until freeze drying (Virtis Advantage EL, Depex) for Aβ ELISA and fatty acid analysis. Freeze dried hemispheres were grinded in a mixer mill (Retsch, MM200) for 45 s at 20 Hz and the tissue was stored at −20°C until further analysis. The right hemisphere was fixed in 4% paraformaldehyde for 24 h and then rinsed in PBS and stored in PBS with 0.1% azide until further processing for histological analyses.

Aβ ELISA

For the human Aβ ELISA assay on brain samples of transgenic mice, 20 mg of grinded freeze dried brain tissue was homogenized in 200 μL PBS (Invitrogen). The homogenate was centrifuged at 13,000 rpm, 10 min at 4°C. The supernatant was removed. The pellet was resuspended in 80 μL 4× formic acid (VWR International) and sonicated for 10 min. The sample was neutralized by adding 720 μL 4 M Tris. After centrifuging (13.000 rpm, 10 min at 4°C), the supernatant was used to detect Aβ, using the hAmyloid β42 Brain ELISA and the hAmyloid β40 Brain ELISA (Genetics Company, TKbrain-Set). According to the manufacturer's protocol, a standard curve was made with a range of 25–500 pg/ml. The samples were diluted 50–100× using the dilution buffer supplied by the manufacturer. 50 μL of antibody conjugate solution was added to the 96 wells plate before 50 μL of standard or sample was added. The plates were covered and mixed thoroughly on a plate shaker. After an overnight incu-

Table 2
Detailed compositions of the experimental diets that were used in experiments A and B. All diets were isoenergetic, contained 5% fat (oli blend specified in Table 3), and also contained standard vitamin and mineral premix, providing recommended daily amounts of these nutrients. Specific nutrients or combinations of nutrients that were supplemented on top of the standard Control diet are indicated in the lower part of the table (UMP through Vit.B12). All amounts of nutrients are indicated in g/100 g of diet. All diets were presented to the animals as pellets

Ingredients (g/100 g diet)	Experiment A		Experiment B			
	Control	FC	Control	DHA	UMP	DHA + UMP
Wheat	25.87	23.82	25.87	25.87	24.87	24.87
Barley	25.00	25.00	25.00	25.00	25.00	25.00
Semolina	25.00	25.00	25.00	25.00	25.00	25.00
Soybean meal	8.50	8.50	8.50	8.50	8.50	8.50
Whey	5.00	5.00	5.00	5.00	5.00	5.00
Bentonite	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin/mineral premix	2.20	2.20	2.20	2.20	2.20	2.20
$CaCO_3$	1.40	1.40	1.40	1.40	1.40	1.40
Dicalciumphosphate	0.30	0.30	0.30	0.30	0.30	0.30
NaCl	0.50	0.50	0.50	0.50	0.50	0.50
L-lysine HCl	0.18	0.18	0.18	0.18	0.18	0.18
DL-methionine	0.05	0.05	0.05	0.05	0.05	0.05
Oil blend	5.00	5.00	5.00	5.00	5.00	5.00
Providing DHA	–	0.757	–	0.757	–	0.757
Providing EPA	–	0.189	–	0.189	–	0.189
UMP	–	1.000	–	–	1.000	1.000
Choline	–	0.313	–	–	–	–
Lecithin	–	0.412	–	–	–	–
Vitamin E	–	0.157	–	–	–	–
Vitamin C	–	0.160	–	–	–	–
Selenium	–	0.000111	–	–	–	–
Folic acid	–	0.0007	–	–	–	–
Vitamin B6	–	0.0027	–	–	–	–
Vitamin B12	–	0.0000011	–	–	–	–

Table 3
Specification of the oil blends that were used for the various diets in experiments A and B. All diets contained 5% of fat. Relative amounts of saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA), and poly-unsaturated fatty acids (PUFA) were similar for all diets

		Experiment A		Experiment B			
		Control	FC	Control	DHA	UMP	DHA UMP
Source	Soy oil	1.39		1.39		1.39	
	Coconut oil	0.65	0.10	0.65	0.10	0.65	0.10
	Corn oil	2.96	1.75	2.96	1.75	2.96	1.75
	Fish oil		3.15		3.15		3.15
%FA	SFA	24	26	24	26	24	26
	MUFA	24	24	24	24	24	24
	PUFA	52	49	52	49	52	49

bation at 4°C, the plates were washed 5 times with the supplied washing buffer. 100 μL of enzyme conjugate was added to wells for 30 min incubation at room temperature. The plate was washed 5 times and subsequently 100 μL of substrate was added. After 25 min incubation in the dark at room temperature, 50 μL of stop solution was added and the plates were measured at 450 nm with a reference filter of 620–650 nm.

Histology

The fixed hemispheres were processed by NeuroScience Associates (Knoxville, USA), who subsequently stained two adjacent series of sections. One series of sections was subjected to an immunohistochemical staining with an antibody for $A\beta_{1\text{-}42}$ to reveal amyloid plaques. Another series of sections was stained with the deOlmos Amino Cupric Silver method to reveal ongoing disintegrative degeneration [25, 26]. In short, hemispheres, arranged in 4×8 arrays, were embedded in a gelatin matrix using MultiBrain® Technology. Each block of embedded hemispheres was rapidly frozen by immersion in isopentane chilled to −70°C with crushed dry ice. Frozen sections (35 μm) were then cut in the coronal plane throughout the striatum-hippocampus part of the

hemispheres. Sections to be stained with the Aβ antibody were stored in antigen preserve storage buffer; the sections to be stained with the Amino Cupric Silver method were stored in Fix-storage buffer.

Image analysis

Images of the stained sections were obtained by using. Slide (Olympus, Zoeterwoude, The Netherlands) and image analyses were performed with AnalySIS Five (Olympus). Using the unbiased sampling method, regions of interest (ROI) were selected in the scanned sections for both the hippocampus and the overlying neocortex. Surface areas were stereologically quantified using the Cavalieri method. Total area stained per ROI was determined by densitometric measurements and expressed as plaque burden ($A\beta_{1\text{-}42}$ staining) or degenerative burden (visualized with deOlmos Amino Cupric Silver stain for disintegrative degeneration), calculated as the ratio of stained surface and total ROI surface for each respective brain area (hippocampus or neocortex).

Brain fatty acid analysis

Fatty acid analyses were performed on 20 mg of the grinded freeze dried brain tissue that was homogenized in 1% EDTA solution. Total lipid content was extracted from the homogenates by adding methanol and dichloromethane. Subsequently, samples were centrifuged at 3000 rpm for 10 min and the lower layer (dichloromethane and lipids) was collected. 200 μl of the dichloromethane layer was evaporated to dryness in a SpeedVac® concentrator. 2 mL methanol and 40 μL concentrated sulfuric acid were added to the dried extract. The samples were heated at 100°C for 60 min, and 2 mL hexane and 0.5 mL 2.5 M sodium hydroxide solution were added. After vortexing and centrifuging the samples for 5 min at 3000 rpm, the upper layer was collected and evaporated in a SpeedVac®. The fatty acids were dissolved in 125 μL iso-octane and analyzed on a GC-FID with a CP-SIL88 column (50 m × 0.25 mm id. 0.22 μm film thickness).

Statistics

All statistical analyses were performed using SPSS 15.0 (SPSS Benelux). Effects of the different diets on body weight were analyzed using repeated measures ANOVA with Weeks having 13 levels as within-subject variable. Between-subject variables in experiment A consisted of Genotype (transgenic and wild type) and/or Diet (Control and FC). In experiment B, the between-subject variable Diet had 4 levels (Control, DHA, UMP, and DHA + UMP). Effects of diets on Aβ ELISA, amyloid plaque burden, and degenerative burden were analyzed using univariate ANOVA. Effects of diets on brain fatty acid profiles were analyzed using multivariate ANOVA. For all analyses, differences were considered significant at $p < 0.05$. *Post hoc* comparisons were performed when appropriate. Next to the primary statistical analyses according to the original experimental design, *a posteriori* exploratory statistical analyses were performed using ANOVA to compare effects of FC diet (experiment A) and DHA + UMP diet (experiment B) on Aβ pathology in transgenic mice.

RESULTS

Body weights

In both experiment A and B, body weights increased over Weeks ($F(12,372) = 79.95$; $p < 0.001$ and $F(12,276) = 94.63$; $p < 0.001$, respectively). No main effect of Genotype, Diet, or their interaction was found to be significant. A significant Genotype x Weeks interaction in experiment A ($F(12,372) = 3.45$; $p < 0.001$) indicated that weight gain over time was larger in wild type mice, irrespective of Diet.

Amyloid and related pathological changes: experiment A

Data obtained in experiment A with respect to brain amyloid parameters are displayed in Fig. 1.

Aβ ELISA

In brain homogenates of transgenic mice, the FC diet significantly reduced both the levels of $A\beta_{42}$ ($F(1,12) = 6.28$; $p < 0.05$; Fig. 1a) and $A\beta_{40}$ ($F(1,12) = 5.81$; $p < 0.05$; Fig. 1b) as compared to Control diet.

Amyloid plaque burden

AβPP/PS1 transgenic mice had a higher amyloid plaque burden in the hippocampus than wild type mice ($F(1,31) = 334.4$; $p < 0.001$; Fig. 1c). The FC diet reduced the amyloid plaque burden in the hippocampus compared to the Control diet ($F(1,31) = 6.28$; $p < 0.02$). A significant Diet x Genotype interaction ($F(1,31) = 6.22$; $p < 0.02$) was found. *Post-hoc* analyses showed no significant effect of Diet in wild type mice ($p = 0.68$). Similar to the hippocampus, the amyloid plaque burden in the neocortex of transgenic mice was

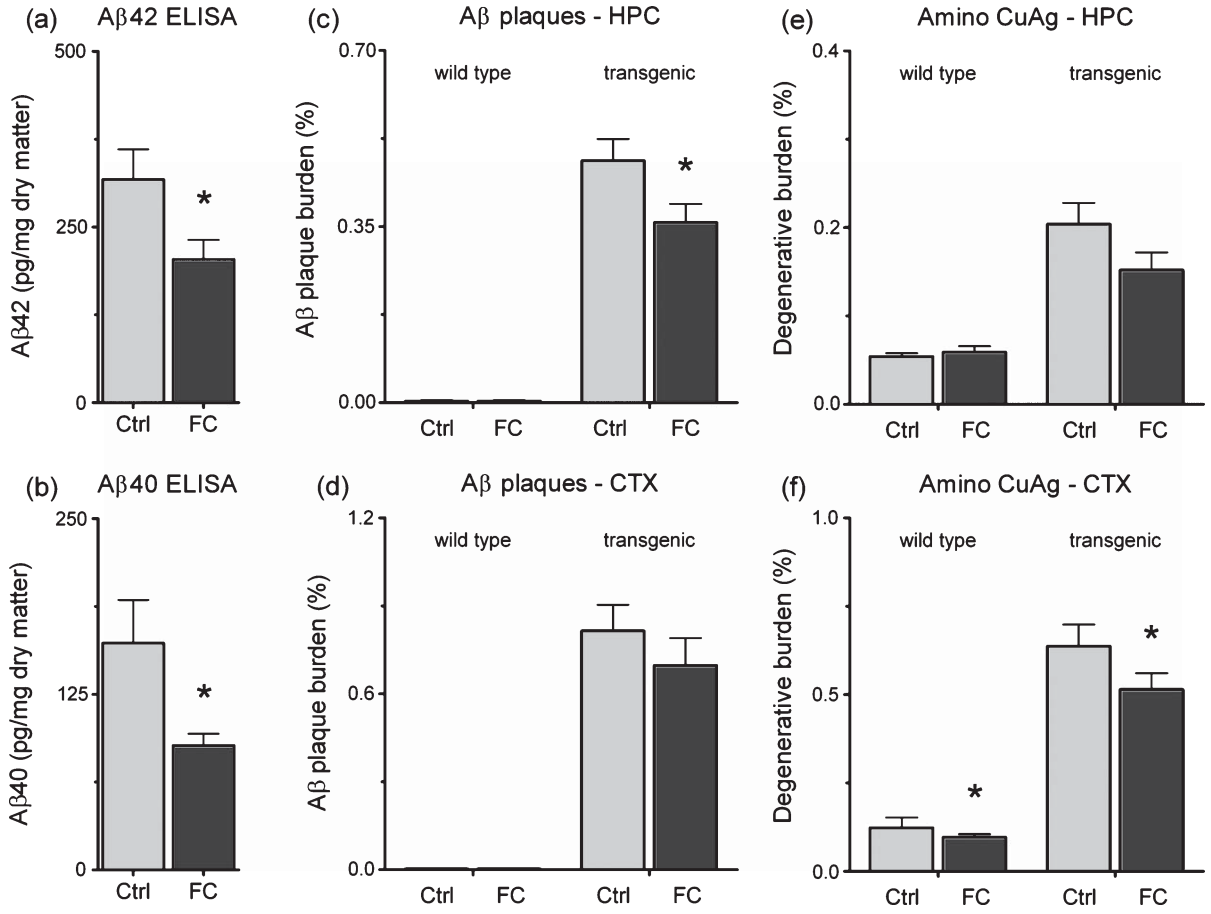

Fig. 1. Effects of Control and FC diets on brain amyloid related parameters in AβPP/PS1 transgenics and their wild type mice in experiment A. All graphs display means (+SEM). Graphs on the left display levels of $A\beta_{42}$ (a) and $A\beta_{40}$ (b) as measured by ELISA in freeze dried brain homogenates from transgenic mice. Graphs in the middle display percentage amyloid plaque burden as indicated by $A\beta_{42}$ staining in the hippocampus (HPC, c) and the neocortex (CTX, d) of wild type and transgenic mice. Graphs on the right display percentage degenerative burden as indicated by Amino Cupric Silver staining in the hippocampus (HPC, e) and the neocortex (CTX, f) of wild type and transgenic mice. Dietary interventions are indicated at the x-axes. $^*p < 0.05$ versus Control.

elevated in comparison to wild types ($F(1,31) = 204.4$; $p < 0.001$; Fig.1d). The FC diet did not affect amyloid plaque burden in the neocortex ($p = 0.48$).

Degenerative burden

Although some ongoing degeneration was present in the brains of all mice, the degenerative burden in transgenic mice was significantly increased in both the hippocampus ($F(1,31) = 97.3$; $p < 0.001$; Fig. 1e) and the neocortex ($F1, 31) = 169.4$; $p < 0.001$; Fig. 1f) as compared to wild type mice. The FC diet did not affect degenerative burden in the hippocampus ($F(1,31) = 1.75$; $p = 0.195$). In the neocortex, however, the FC diet induced a significant decrease in degenerative burden ($F(1,31) = 4.44$; $p < 0.05$), irrespective of Genotype.

Representative illustrations of plaque burden (Aβ) and degenerative burden (Amino CuAg) in the brains of animals from experiment A are given in Fig. 2.

Amyloid and related pathological changes: experiment B

Data obtained in experiment B with respect to brain amyloid parameters are displayed in Fig. 3.

Aβ ELISA

The dietary interventions affected brain levels of $A\beta_{42}$ ($F(3,23) = 3.67$; $p < 0.05$; Fig. 3a) and $A\beta_{40}$ ($F(3,23) = 3.63$; $p < 0.05$; Fig. 3b). Levels of $A\beta_{42}$ in the DHA + UMP group were higher than those in the DHA and UMP groups (both $p < 0.05$). Levels of $A\beta_{40}$

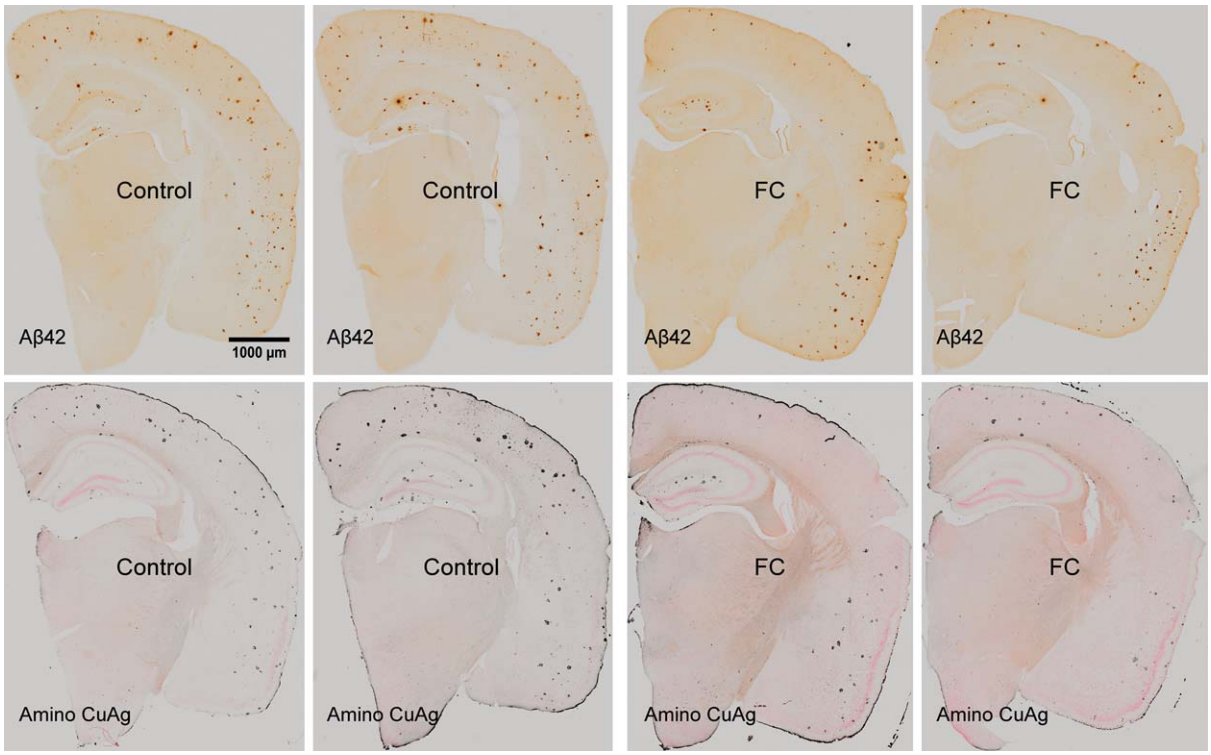

Fig. 2. Representative images of $A\beta_{42}$ (Aβ; top row) and deOlmos Amino Cupric Silver (Amino CuAg; bottom row) staining in brain sections from AβPP/PS1 mice of experiment A. Images on the left show amyloid plaques and associated degenerative staining in adjacent sections in a mouse on the Control diet. Images on the right are taken from adjacent sections in a mouse on the FC diet. Group means of plaque burden and degenerative burden in hippocampus and neocortex are shown in Fig. 1c–f. The FC diet reduced plaque burden in the hippocampus and degenerative burden in the neocortex.

were reduced by the UMP diet as compared to both the Control and the DHA + UMP diets (both $p < 0.05$).

Amyloid plaque burden

Dietary interventions with DHA, UMP, or DHA + UMP in AβPP/PS1 transgenic mice did not induce significant changes in amyloid plaque burden as compared to the Control diet in either the hippocampus ($F(3,23) = 1.16$; $p = 0.35$; Fig. 3c) or the neocortex ($F(3,23) = 1.36$; $p = 0.28$; Fig. 3d). Representative illustrations of plaque burden in the brains of animals from experiment B are given in Fig. 4.

Degenerative burden

Degenerative burden in the hippocampus was not significantly affected by dietary interventions with DHA, UMP, or DHA + UMP in AβPP/PS1 transgenic mice ($F(3,23) = 2.09$; $p = 0.129$; Fig. 3e). Similarly, the latter diets did not affect degenerative burden in the neocortex ($F(3,23) = 0.80$; $p = 0.506$; Fig. 3f).

A posteriori exploratory comparisons: experiments A and B

Next to the primary statistical analyses according to the original experimental design, it was decided to perform some *a posteriori* exploratory statistical analyses. For the measures related to Aβ pathology in transgenic mice, the effects of the FC diet in experiment A were directly compared to the effects of the DHA + UMP diet in experiment B. These comparisons were considered relevant in the light of the original aim concerning potential synergistic actions of nutrients. The results of the analyses indicated that, compared to the DHA + UMP diet, the FC diet resulted in significantly lower levels of $A\beta_{42}$ ($F(1,12) = 11.20$; $p < 0.01$) and $A\beta_{40}$ ($F(1,12) = 50.19$; $p < 0.001$), and a lower amyloid plaque burden in the hippocampus ($F(1,12) = 5.02$; $p < 0.05$) but not in the neocortex ($p = 0.36$). As compared to the DHA + UMP diet, the FC diet resulted in significantly lower degenerative burden in the hippocampus ($F(1,12) = 6.33$; $p < 0.05$)

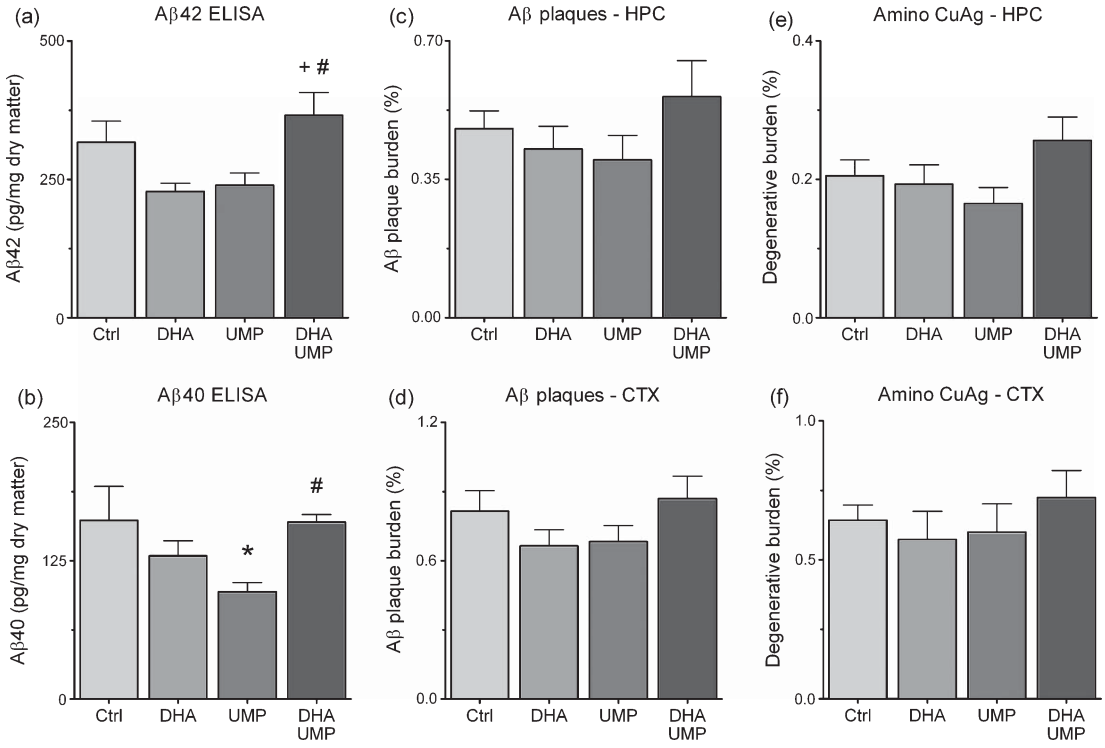

Fig. 3. Effects of Control, DHA, UMP, and DHA + UMP diets on brain amyloid related parameters in AβPP/PS1 transgenics mice in experiment B. All graphs display means (+SEM). Graphs on the left display levels of $A\beta_{42}$ (a) and $A\beta_{40}$ (b) as measured by ELISA in freeze dried brain homogenates. Graphs in the middle display percentage amyloid plaque burden as indicated by $A\beta_{42}$ staining in the hippocampus (HPC, c) and the neocortex (CTX, d). Graphs on the right display percentage degenerative burden as indicated by Amino Cupric Silver staining in the hippocampus (HPC, e) and the neocortex (CTX, f). Dietary interventions are indicated at the x-axes. $^{*}p < 0.05$ versus Control; $^{+}p < 0.05$ versus DHA; $^{\#}p < 0.05$ versus UMP.

and marginally lower degenerative burden in the neocortex ($F(1,12) = 3.82$; $p = 0.07$).

Brain fatty acids

The relative amounts of different fatty acids in the lipid fraction of the brain homogenates from experiment A and B are displayed in Tables 4 and 5, respectively.

In experiment A (Table 4), small differences between brain fatty acid profiles of AβPP/PS1 transgenic and wild type mice were noted. Main effects of Genotype were found for 18:0 (stearic acid; $F(1,31) = 5.31$; $p < 0.05$), 18 : 1n9 (oleic acid; $F(1,31) = 2.90$; $p < 0.05$), and total PUFAs ($F(1,31) = 4.23$; $p < 0.05$). For 22 : 6n3 (DHA) the Diet × Genotype interaction just failed to reach significance ($F(1,31) = 3.24$; $p = 0.08$), indicating that the Diet-induced increase in DHA tended to be larger in transgenic mice. The FC diet reduced n6 PUFAs ($F(1,31) = 575.86$; $p < 0.001$) and increased n3 PUFAs ($F(1,31) = 125.42$; $p < 0.001$) relative to the Control diet in both AβPP/PS1 transgenic and wild type mice. The FC diet decreased the relative amount of 20 : 4n6 (AA; $F(1, 31) = 626.12$; $p < 0.001$) and increased DHA ($F(1,31) = 694.37$; $p < 0.001$). The FC diet decreased the total relative amount of PUFAs ($F(1,31) = 8.00$; $p < 0.01$). The total relative amount of MUFAs was increased by the FC diet ($F(1,31) = 16.30$; $p < 0.001$), mainly due to an increase in 18 : 1n9 ($F(1,31) = 88.39$; $p < 0.001$).

In experiment B (Table 5), the DHA diet and the DHA + UMP diet similarly affected brain fatty acid profiles as compared to both the Control diet and the UMP diet. Both DHA-containing diets decreased 18:0 ($F(3,23) = 3.14$; $p < 0.05$), and increased both 18:1n9 ($F(3,23) = 18.27$; $p < 0.001$) and the total relative amount of MUFAs ($F(3,23) = 3.49$; $p < 0.05$). The DHA-containing diets decreased the n6 PUFAs ($F(3,23) = 178.01$; $p < 0.001$) including AA

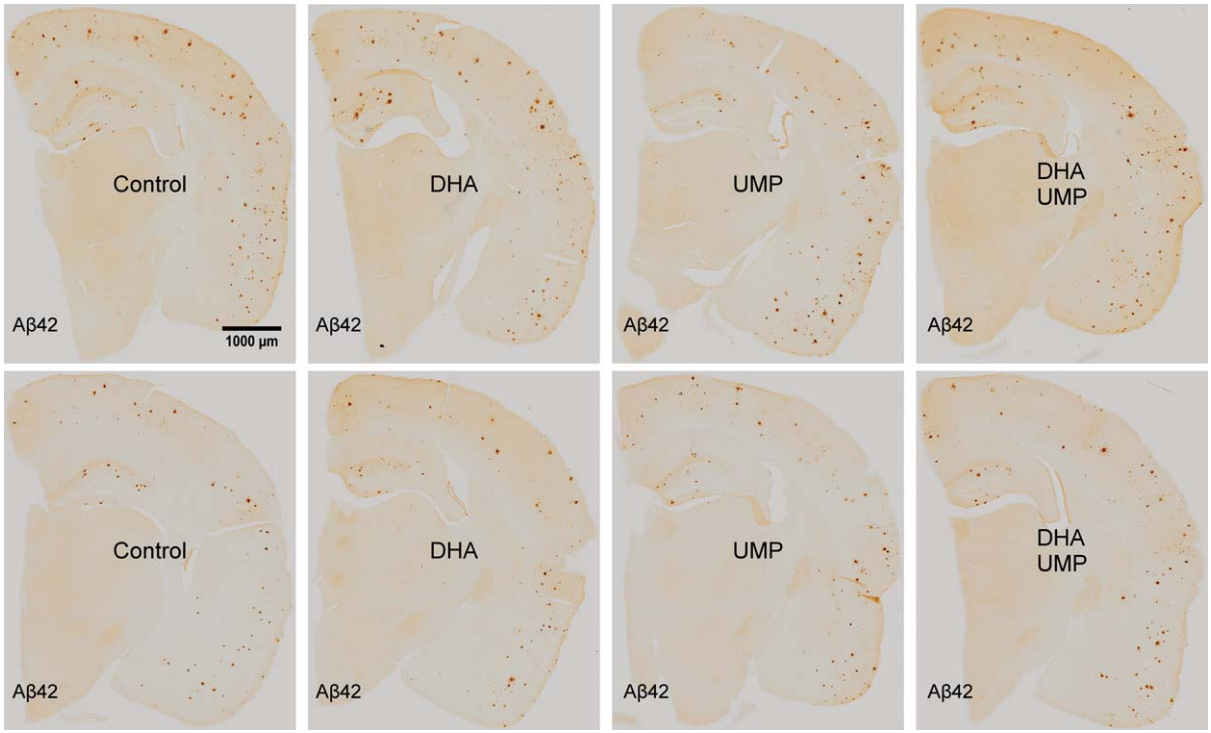

Fig. 4. Representative images of $A\beta_{42}$ (Aβ) staining in brain sections from AβPP/PS1 mice of experiment B. From left to right, images from mice on the Control diet, the DHA only diet, the UMP only diet, and the DHA + UMP diet are presented. Group means of plaque burden in hippocampus and neocortex, as shown in Fig. 3c, d, were not significantly different for the Diet groups.

Table 4
Relative amounts of the major individual fatty acids and their classes in the lipid fraction of the brain homogenates from experiment A. Data are expressed as means, with SEMs within parentheses. Statistically significant main effects ($p < 0.05$) of Genotype (G) and Diets (D) are indicated at the right hand side; trends ($p < 0.10$) are indicated within parentheses

Expt. A	Wild type		Transgenic		Main
	Control	FC	Control	FC	effects
16 : 0	20.37 (0.11)	20.50 (0.16)	20.19 (0.13)	20.47 (0.12)	
18 : 0	16.69 (0.07)	16.45 (0.14)	16.95 (0.17)	16.77 (0.12)	G
18 : 1n9	15.27 (0.08)	16.26 (0.12)	15.08 (0.12)	16.02 (0.08)	D,G
20 : 4n6	8.35 (0.08)	6.32 (0.08)	8.34 (0.06)	6.47 (0.05)	D
22 : 6n3	12.43 (0.14)	13.85 (0.18)	12.43 (0.13)	14.44 (0.17)	D,(D × G)
SFA	39.26 (0.12)	39.06 (0.30)	39.38 (0.28)	39.19 (0.20)	
MUFA	24.01 (0.22)	25.07 (0.22)	23.82 (0.21)	24.53 (0.17)	D
PUFA	25.71 (0.23)	24.82 (0.21)	25.92 (0.22)	25.53 (0.18)	D,G
n6	12.73 (0.12)	9.84 (0.10)	12.95 (0.15)	9.94 (0.11)	D
n3	12.98 (0.18)	14.98 (0.22)	12.97 (0.19)	15.60 (0.19)	D

($F(3,23) = 139.00$; $p < 0.001$), while they increased the n3 PUFAs ($F(3,23) = 68.29$; $p < 0.001$) including DHA ($F(3,23) = 58.93$; $p < 0.001$).

DISCUSSION

The present results indicate not only that dietary intervention can affect AD-like pathology in transgenic AβPP/PS1 mice, but also that dietary composition, i.e., the simultaneous presence of specific nutrients, is an important factor in determining their efficacy. The specific multi-nutrient enriched FC diet was shown to reduce several pathology-related measures, including total brain $A\beta_{42}$ and $A\beta_{40}$ levels as measured by ELISA. In addition, by applying immunohistochemical techniques and a stereological approach to determine brain plaque burden, it was shown that

Table 5
Relative amounts of the major individual fatty acids and their classes in the lipid fraction of the brain homogenates from experiment B. Data are expressed as means, with SEMs within parentheses. Statistically significant main effects of Diets (D) are indicated at the right hand side. Post-hoc comparisons indicated that both DHA-containing diets (DHA and DHA + UMP) differed from the remaining diets (Control and UMP)

Expt. B	Transgenic				Main
	Control	DHA	UMP	DHA UMP	effects
16 : 0	20.19 (0.13)	20.45 (0.21)	20.69 (0.26)	20.38 (0.21)	
18 : 0	16.95 (0.17)	16.64 (0.11)	17.20 (0.19)	16.62 (0.10)	D
18 : 1n9	15.08 (0.12)	16.00 (0.21)	14.96 (0.07)	15.97 (0.14)	D
20 : 4n6	8.34 (0.06)	6.50 (0.13)	8.38 (0.07)	6.45 (0.11)	D
22 : 6n3	12.43 (0.13)	14.40 (0.22)	12.48 (0.12)	14.59 (0.17)	D
SFA	39.38 (0.28)	39.11 (0.28)	39.97 (0.40)	39.04 (0.27)	
MUFA	23.82 (0.21)	24.48 (0.42)	23.55 (0.15)	24.55 (0.32)	D
PUFA	25.92 (0.22)	25.57 (0.26)	25.99 (0.14)	25.56 (0.23)	
n6	12.95 (0.15)	9.99 (0.18)	12.93 (0.09)	9.88 (0.12)	D
n3	12.97 (0.19)	15.58 (0.23)	13.06 (0.12)	15.67 (0.20)	D

this multi-nutrient enrichment affected both amyloid plaque burden and degenerative burden in different brain structures. FC-induced reductions reached statistical significance for amyloid plaque burden in the hippocampus and for degenerative burden in the surrounding neocortex. These findings are in line with and extend our recent findings obtained in the Aβ infusion model [21], by showing that FC diet may not only affect the Aβ-induced membrane toxicity and neurodegeneration, but also the membrane-bound production of Aβ. In contrast, diets that were only enriched in DHA, UMP, or their combination yielded non-significant changes in amyloid plaque burden and degenerative burden in hippocampus and neocortex in these mice, although the UMP diet did reduce brain $A\beta_{40}$ levels. It should be noted that the DHA diet and the DHA + UMP diet were equally effective in raising brain DHA levels as the FC diet. Together these data indicate that the multi-nutrient enrichment by FC is effective in reducing pathological changes in the AβPP/PS1 model, and that the effects of this dietary intervention are not easily attributed to one or more of its individual components.

Since especially some animals on the DHA diet died over the course of the study, this group was left with a relative small number of subjects. It is possible that this may have hampered us to observe statistically significant effects of the diet on AD-like pathology compared to Controls. Nevertheless, in experiment B the largest differences were found between the DHA + UMP diet and the DHA or UMP only diets, underlining the importance of dietary context. This importance of dietary context was further supported by the results of the *a posteriori* exploratory analyses showing that the FC diet was more effective in reducing Aβ pathology than the combination of nutrients in the DHA + UMP diet.

Previously, various studies have examined the effects of single-nutrient dietary supplementation on brain amyloid in different transgenic mouse models of AD. A number of these studies focused on the effects of supplementation of DHA or DHA-containing fish oil, with variable outcome. In an early study, Lim and coworkers [43] reported that a high DHA diet significantly decreased amyloid burden in aged Tg2576 mice, but only as compared to a DHA-depleting diet. In 3xTg-AD mice, dietary supplementation of DHA reduced soluble, but not insoluble Aβ levels using whole-brain homogenates [39]. However, a more recent study found no significant effects of DHA consumption on Aβ pathologies in frontal cortex samples of 3xTg-AD mice [41]. Similarly, the present findings obtained with DHA in experiment B are not at variance with previous observations in the AβPP/PS1 model. In this model, some reductions of hippocampal Aβ levels and/or hippocampal plaque load induced by fish oil containing diets have been reported [24], albeit not consistently [40]. In a series of experiments using long term dietary interventions in the AβPP/PS1 model, it was shown that fish oil may reduce vascular Aβ in the neocortex more readily than parenchymal Aβ plaques in the hippocampus [44]. In addition, it was shown that the effectiveness of fish oil diets on reducing neocortical Aβ plaque load in this model may depend on both the age at which the intervention is started and on the length of the intervention [45]. Importantly, the present data indicate that the effectiveness of fish oil containing diets also depends on the concomitant availability of additional nutrients. This observation is in line with a recent report suggesting that the influence of DHA

on amyloid plaque formation may depend upon the composition of the diet in which it is supplied [42].

To date, the effects of UMP administration on AD-like pathology in mouse models have less extensively been investigated than those of DHA. Nevertheless, the effects of UMP in experiment B were more pronounced than those of DHA and reached statistical significance for brain $A\beta_{40}$ levels. The underlying mechanism by which UMP may affect Aβ production is unknown, but might involve changes in AβPP processing. Oral intake of UMP has been shown to increase brain levels of UTP [46] that may activate brain P2Y receptors [47, 48]. *In vitro* studies have shown that P2Y2 receptor activation by UTP resulted in enhanced non-amyloidogenic processing of AβPP [49]. Such a shift in AβPP processing would be expected to result in decreased Aβ release.

In view of the separate effects of DHA and UMP observed in experiment B, the results obtained with combined administration of DHA + UMP are surprising. Thus, whereas UMP reduced brain $A\beta_{40}$ levels as compared to Control diet, the combination of DHA + UMP induced an increase in $A\beta_{40}$ as compared to UMP diet. Similarly, the combination of DHA + UMP induced an increase in brain $A\beta_{42}$ levels as compared to the DHA diet and the UMP diet, while non-significant reductions in brain $A\beta_{42}$ levels were observed with the single-nutrient diets. Similar tendencies were observed for plaque burden and degenerative burden in the hippocampus and the neocortex. These seemingly contrasting findings are not easily explained. However, the difference in effect on amyloid pathology induced by the DHA diet, the DHA + UMP diet, and the FC diet demonstrates that the effects of these nutritional interventions are not the mere sum of the effects of their individual components. Interestingly, all these three DHA-containing diets induced very similar changes in brain fatty acid profiles, with increases in DHA and oleic acid, and decreases in AA, despite differences in effects on pathology. First, these data show that increased brain levels of DHA are not sufficient to decrease Aβ production, amyloid plaque burden and degeneration in AβPP/PS1 mice. Second, these data indicate that the combined administration of nutrients may yield seemingly unexpected cumulative actions. Co-administration of the dietary precursors for phospholipid synthesis DHA, UMP, and choline has previously been shown to stimulate neuronal membrane synthesis and dendritic spine formation more than enrichment of a single precursor [16]. Whether the stimulation of membrane synthesis and spine formation may have increased AβPP processing and subsequent Aβ production in the AβPP/PS1 mice remains to be determined. Third, these data indicate that nutrients present in FC other than DHA and UMP contribute significantly to the overall effect of this multi-nutrient mixture on AD-like pathology. Indeed, several studies using single nutrient enrichment provide some, albeit weak, support for the potential beneficial effects of these nutrients. For instance, in line with the idea that antioxidants like vitamins E and C may improve the neuroprotective effects of DHA [50], vitamin E was shown to help reduce Aβ levels and amyloid deposition in young Tg2576 mice [51], although combined administration of vitamins E and C did not reduce amyloid deposition in AβPP/PS1 mice [52]. Similarly, B-vitamin deficiency has sometimes been found to increase Aβ levels and deposition in Tg2576 mice [53], but not always [54]. The effects of combined B-vitamins on Aβ production may result from changes in β- and γ-secretase activity [55]. In this respect there is an overlap in modes of action with those of nutrients like DHA [37], which may help to understand the efficacy of a multi-nutrient approach. An elegant example of the concerted action of the nutrients in FC on membrane bound G-protein coupled receptor functioning is provided by Savelkoul et al. [56].

In line with previous reports [22, 23, 57], we observed higher brain levels of $A\beta_{42}$ than $A\beta_{40}$ in young adult AβPP/PS1 animals of 6 months. Using an $A\beta_{42}$ antibody for immunohistochemistry, we also noted at this stage an anatomical differentiation in the development of amyloid plaques, showing higher levels of amyloid plaque burden in the neocortex than in the hippocampus. In the AβPP/PS1 mice, degenerative staining displayed a similar anatomical differentiation and a close overlap with amyloid staining, supporting a link between $A\beta_{42}$ levels, amyloid plaque formation and neurotoxicity in these mice. In wild type mice we also observed some degenerative staining, and interestingly, the FC diet reduced neocortical neurodegeneration in wild type and transgenic animals to a similar extent, suggesting that the diet may have protective effects irrespective whether the degeneration is caused by amyloid toxicity.

The present effects obtained with the multi-nutrient enriched FC diet confirm and extend recent observations in the Aβ infusion model [21], showing that the same FC diet reduced both the neuroanatomical and behavioral consequences of Aβ toxicity. We now show that this multi-nutrient enrichment effectively reduces Aβ production and AD-like pathology in the AβPP/PS1 mouse model, while enrichment with DHA, UMP, or their combination had little effect. Whether

the observed reductions in pathology may contribute to the preservation of cognitive performance remains to be determined. It may however be speculated that the presently observed reductions in Aβ production and amyloid-related neurodegeneration may be part of the mechanisms underlying observed improvements of memory performance following this multi-nutrient intervention in mild AD patients [58, 59]. Additional studies investigating the effects of longer term dietary interventions on cognitive performance in AβPP/PS1 mice, AD patients, and subjects with prodromal AD are currently ongoing.

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